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Abstract: The current understanding about ethanol effects on the ligand-gated ion channel (LGIC) superfamily has been restricted to identify potential binding sites within transmembrane (TM) domains in the Cys-loop family. Here, we demonstrate a key role of the TM3–4 intracellular loop and G$\beta\gamma$ signaling for potentiation of glycine receptors (GlyRs) by ethanol. We discovered 2 motifs within the large intracellular loop of the GlyR α_1 subunit that are critical for the actions of pharmacological concentrations of ethanol. Significantly, the sites were ethanol-specific because they did not alter the sensitivity to general anesthetics, neurosteroids, or longer *n*-alcohols. Furthermore, G$\beta\gamma$ scavengers selectively attenuated the ethanol effects on recombinant and native neuronal GlyRs. These results show a selective mechanism for low-ethanol concentration effects on the GlyR and provide a mechanism on ethanol pharmacology, which may be applicable to other LGIC members. Moreover, these data provide an opportunity to develop new genetically modified animal models and novel drugs to treat alcohol-related medical concerns.

Keywords: pharmacology, signal transduction, glycine receptor, alcoholism, G proteins

Ethanol has been the most widely abused drug throughout mankind's history. Its consumption at pharmacological doses produces strong modifications in motor, sensorial, and cognitive functions, which lead to great economical and social consequences. Unlike marihuana and morphine, which are linked to specific G protein-coupled receptors, ethanol modifies excitability by affecting a large number of cellular effectors. A number of electrophysiological studies have demonstrated that ethanol can modulate the activity of several ligand-gated ion channels (LGIC), including members of the Cys-loop family, composed of nicotinic acetylcholine (nAChR), serotonin (5-HT_3R), γ-aminobutyric acid ($GABA_A$R), and glycine receptors (GlyR) (1–3). Because these receptors mediate fast synaptic transmission in the mammalian central nervous system, their alterations by ethanol might explain its complex actions on central nervous system functions.

Inhibitory GlyRs, mostly restricted to spinal cord and brainstem, are critical for the control of excitability of neuronal networks that modulates motor control, respiration, and pain (4–6). GlyRs are composed of 5 subunits in a pentameric quaternary structure arranged around a central pore. Each subunit possesses 4 transmembrane domains (TM) and a large intracellular loop between TM3 and TM4 (4, 5, 7). Binding of glycine induces an increase in Cl^- ion conductance, hyperpolarizing the cell membrane. The GlyR inhibitory activity can be

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modulated by several ligands that include toxins, general anesthetics, and alcohols (4, 5). Previous studies in different cell types have demonstrated that millimolar concentrations of ethanol can enhance the glycine-activated current (4, 5, 8–10). However, the molecular mechanisms by which ethanol modifies this receptor are not well understood. It was reported that mutations in TM residues (S267 and A288) abolished the effect of ethanol (200 mM) on the receptor (8), suggesting that specific amino acids form discrete binding sites that were shared by alcohol and general anesthetics (8, 11). More recent studies, however, have indicated that mutations in these residues interfered with GlyR gating, complicating the interpretation of these results (12). However, several studies indicate that ethanol modulates LGIC activity by indirect effects. For instance, the sensitivity of GlyR to ethanol was affected by intracellular signaling, such as G proteins and kinases (9, 13, 14). In addition, recent studies have provided additional support for the idea that ethanol, at low concentrations, can modulate specific intracellular transduction pathways (15–17). Therefore, because the large intracellular loop of the GlyR can transduce intracellular signaling initiated by PKA, PKC and Gβγ dimers (4–6, 18), it is possible to postulate the existence of discrete molecular determinants for ethanol sensitivity within its structure. Pharmacologically, GlyR potentiation by ethanol might be related to acute intoxication, altering motor and respiratory rhythms (10, 19). Additionally, a new role of accumbal GlyRs on ethanol intake, and potential implications in alcoholism, was recently proposed (20, 21).

In the present work, by using a combination of electrophysiological and molecular techniques in recombinant and native receptors, we describe a function for the large intracellular loop of GlyR for ethanol responsiveness. Strikingly, this Gβγ-linked mechanism was selective for ethanol because it did not alter the receptor sensitivity to other modulators, such as general anesthetics and longer *n*-alcohols. Our results show a selective intracellular mechanism that explains the ethanol effects on a LGIC member and provide key information for the generation of genetically modified animal models and the development of molecules that might block ethanol effects mediated by GlyRs.

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Results

Molecular Sites for Ethanol Potentiation Within the Large Intracellular Loop of the Human GlyR.

We predicted that if the potentiation of GlyR by ethanol depends on signal transduction, mutations in intracellular residues should affect this allosteric effect. Therefore, we performed a functional screening of the human mutant α_1 GlyR by using patch-clamp electrophysiology in transfected HEK293 cells. The cytosolic polypeptide loop containing ≈84 aa has a topology sequence, signal transduction motifs, and presents alternative splicing (4, 5, 22) (Fig. 1*A*). We first examined the sensitivity to ethanol of a GlyR splice variant lacking residues between E326 and K355 (22). Similar to previous studies (4, 5, 8–10), the amplitude of the glycine-activated current in wild-type GlyRs was consistently enhanced by 100 mM ethanol (53 ± 6%, n = 18) (Fig. 1*B*). A comparable response was found in the 326–355 truncated GlyR (54 ± 8%, n = 8) (Fig. 1*B*), indicating that this whole sequence is not important for ethanol potentiation. Interestingly, mutations in a cluster of basic amino acids (^{316}RFRRK) significantly changed the phenotypic property of the receptor making it "ethanol-resistant" (7 ± 3%, n = 14). However, mutations in the residues flanking this sequence did not change sensitivity to ethanol. Consequently, we examined the functional importance of the residues downstream from position K355. The data show that residues between K355 and F380 are not important (Fig. 1*B*). Nevertheless, replacing the sequence ^{381}IQRAKK to alanines again converted the receptor to an ethanol-resistant phenotype. Interestingly, further mutations within this sequence showed the importance of 2 basic residues (^{385}KK) that significantly attenuated the effect of 100 mM ethanol (9 ± 3%, n = 13, Fig. 1*B*). To determine whether specific amino acids were involved in the alcohol effects, we carried out additional analyses in these 2 regions. Within the ^{316}RFRRK cluster, all of the double and single mutations showed partial attenuations in the sensitivity to ethanol (Fig. 1*C*). However, single mutations in the lysine motif ^{385}KK also changed the ethanol sensitivity, indicating important roles of these residues for the ethanol effects on the GlyR (Fig. 1*C*). In addition, we tested a wide range of alcohol concentrations (1–200 mM) on the 316–320A and 385–386A mutants and found that these residues are important for ethanol

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potentiation at behaviorally relevant concentrations (1–100 mM) (Fig. 1 *D* and *E*). However, these mutations did not affect the potentiation elicited by 500 mM ethanol (Fig. 1*F*), suggesting that the identified basic residues are only important for ethanol effects at pharmacological concentrations.

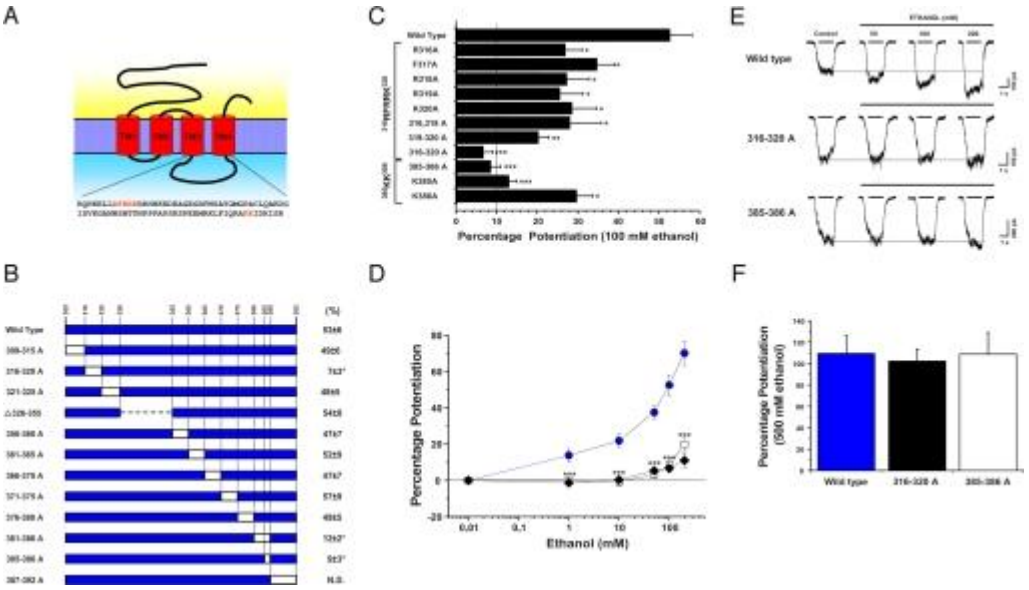

Fig. 1. Molecular sites for ethanol action in the large intracellular loop of the human GlyR. (*A*) Representation of the α_1 GlyR topology and its large intracellular loop primary sequence. Important residues are shown in red. (*B*) Alanine scanning of the GlyR intracellular loop from residues 309 to 392. The white boxes indicate consecutive alanine replacements, and the segmented line depicts a deleted region. The values are the percentages of current potentiation obtained after 100 mM ethanol. The 387–392A mutant (N.D.) did not express a functional channel. (*C*) Sensitivity to 100 mM ethanol of wild-type and mutant GlyRs expressed in HEK293 cells. (*D*) Concentration–response curves to ethanol (1–200 mM) in wild-type (blue circles) and the mutants 316–320A (black diamonds) and 385–386A (white squares). (*E*) Examples of current traces in the presence or absence of ethanol from wild-type and mutant GlyRs. (*F*) Summary of the percentage potentiation elicited after the application of 500 mM ethanol on wild-type (blue) and 316–320A (black) and 385–386A (white) mutants. For all panels, the results are mean ±SEM from normalized glycine-activated currents from 6–17 cells. Differences were significant (*, $P < 0.05$;**, $P < 0.01$;***, $P < 0.001$, ANOVA).

To characterize further the ethanol effects on wild-type and mutant GlyRs, we carried out a set of single-channel experiments by using the outside-out configuration. Previous studies in membranes with mutated TM residues showed that channel gating in ethanol-resistant GlyRs was anomalous, with altered openings and bursts (12). However, the present results showed that both wild-type and 385–386A mutant GlyRs exhibited very similar channel function profiles (Fig. 2*A*), displaying bursts of channel openings with stable

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conductance levels, similar to those shown by other groups (4, 5, 23). Significantly, 10 mM ethanol application strongly modulated wild-type GlyRs, producing a significant enhancement of the open-channel probability (121 ± 22% above control, n = 5) without changes in the main conductance (Fig. 2*A*). Interestingly, the 385–386A mutant was not modulated (−23 ± 23%, n = 5), in agreement with the results obtained by using the whole-cell configuration (Fig. 2 *A* and *B*). Further analysis showed that the main-channel conductance displayed by wild-type and 385–386A mutant GlyRs was not significantly different from those shown in previous reports using wild-type α_1 GlyRs (23) (Fig. 2*C*). Thus, these results demonstrate that mutations in intracellular sites did not cause marked effects on GlyR channel function (Table S1 and Fig. S1 and Fig. S2), but they modified the sensitivity of the receptor to ethanol. The larger effect of ethanol in membrane patches compared with intact cells could be caused by a reduced availability of binding of ethanol to hydrophobic pockets in other targets (11) and the absence of a still unidentified regulatory cytoplasmatic protein that keeps the ethanol effect in check. In line with this finding, a larger Gβγ effect on cell patches compared with whole-cell recordings was reported by our and other groups (24, 25).

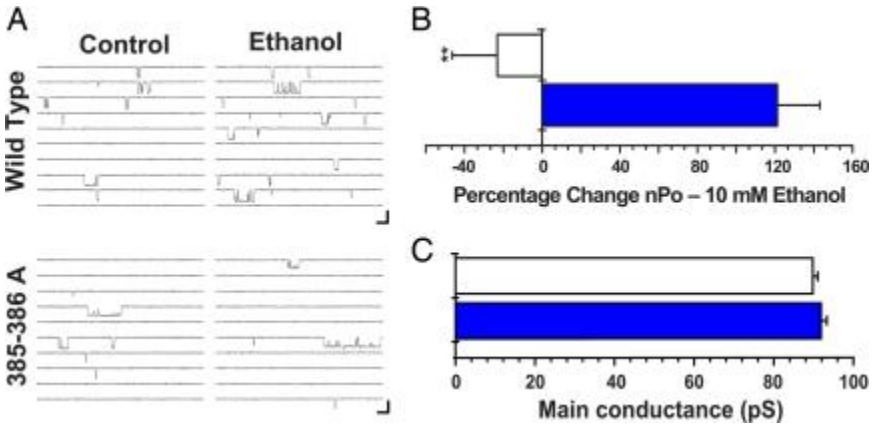

Fig. 2.
Effect of ethanol on single-channel activity in wild-type and 385–386A mutant GlyRs. (*A*) Single-channel recordings from wild-type and 385–386A GlyRs before and after the application of 10 mM ethanol. (Scale bar: 5 pA, 10 ms.) (*B*) The bar graph summarizes the percentage change of open probability after the application of 10 mM ethanol. Differences from WT were significant (**, $P < 0.01$, ANOVA). (*C*) The graph shows that the GlyR mean conductance was not affected by alanine substitutions within the ^{385}KK motif. For all panels, wild-type and the 385–386A mutant are shown in blue and white, respectively.

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Pharmacology of Ethanol-Resistant Mutants to Other GlyR Allosteric Modulators.

It has been proposed that ethanol binds to sites in transmembranes TM2 and TM3 of the GlyR (8, 11). Also, these sites seem to control the efficacy of other allosteric modulators on GlyRs and $GABA_A$ receptors, suggesting similar sites and mechanisms of actions despite their marked structural and physicochemical differences (8, 26–28). Indicating a different mechanism, we found that the ethanol-resistant mutants (316–320A and 385–386A) GlyRs were still nicely modulated by propofol and isoflurane (Fig. 3 *A* and *B*). For example, propofol potentiated the current to 238 ± 15% (n = 12) in wild-type receptors, to 251 ± 24% (n = 9) in the 316–320A GlyR and to 232 ± 35% (n = 6) in the 385–386A mutant. Moreover, the effects of the synthetic neurosteroid alphaxalone, trichloroethanol, and zinc were also similar in wild-type and mutant GlyRs (Fig. 3*C*), demonstrating a high specificity of these residues to ethanol (8, 26–28). Finally, we examined the sensitivity of 316–320A and 385–386A mutants to other *n*-alcohols having longer carbon chains to further test the existence of a binding pocket with spatial restraints for alcohol molecules (11, 28, 29). The data show that both mutant receptors displayed unchanged sensitivities to *n*-alcohols compared with the wild-type GlyR (*SI Text* and Fig. S3). For example, 20 mM butanol enhanced the glycine current in the 316–320A (124 ± 8%, n = 7) and the 385–386A (152 ± 19%, n = 7) mutants to a degree similar to wild-type receptors (126 ± 13%, n = 6) (Fig. 3*D*). Unlike TM mutations that showed modifications in their "cutoff" profiles (29), the intracellular mutants displayed a cutoff at the level of decanol, similar to wild-type (Fig. 3*E*). Altogether, these data show that these intracellular sites can discriminate between pharmacological ethanol concentrations and other modulators, suggesting the presence of a distinct mechanism for the ethanol potentiation of GlyRs.

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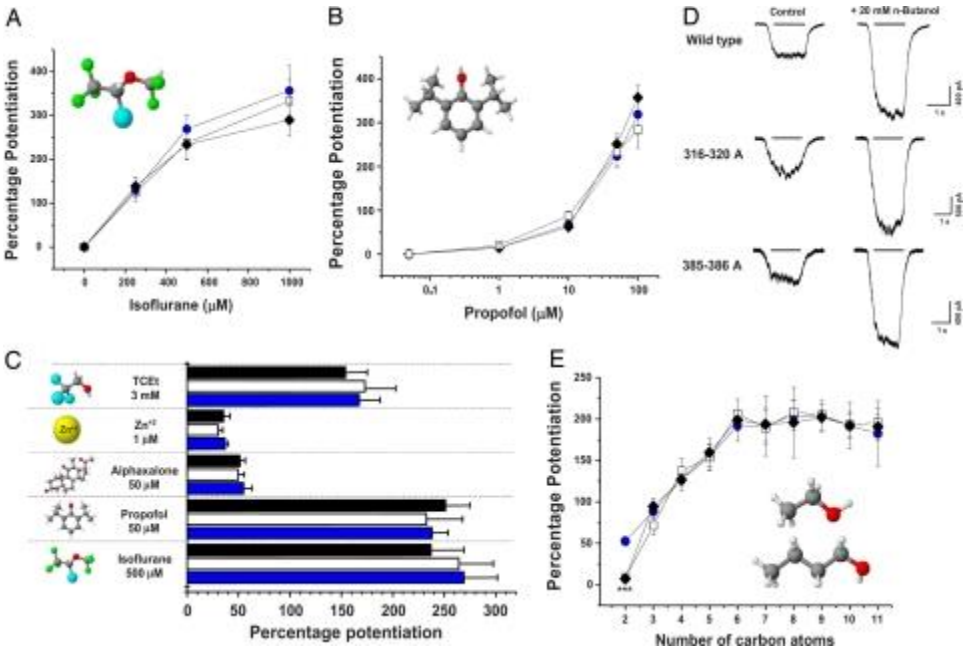

Fig. 3. Pharmacological profiles of mutant GlyRs displaying ethanol-resistant phenotypes. (*A*) Effects of the volatile anesthetic isoflurane on glycine-activated currents elicited by wild-type and mutant GlyRs. (*B*) Concentration-response curves to i.v. anesthetic propofol in wild-type and the mutants 316–320A and 385–386A. (*C*) The graph summarizes the percentage potentiation during application of 3 mM trichloroethanol (TCEt), 1 μM Zn^{2+}, 50 μM alphaxolone, 50 μM propofol, and 500 μM isoflurane on wild-type (blue) and 316–320A (black) and 385–386A (white) mutant GlyRs. (*D*) Glycine-activated current traces from wild-type and mutant GlyRs in the presence of 20 mM butanol. (*E*) The graph summarizes the effects of 100 mM ethanol, 50 mM propanol, 20 mM butanol, 2 mM pentanol, 1 mM hexanol, 0.3 mM heptanol, 0.1 mM octanol, and 50 μM nonanol, decanol, and undecanol on the glycine-activated currents. Chemical structures for ethanol and butanol are shown. For all of the panels, wild-type GlyRs are shown in blue circles, and mutant GlyRs 316–320A and 385–386A are shown in black diamonds and white squares, respectively. The data are presented as mean ±SEM percentage potentiation induced by each modulator. For statistical analysis, at least 6 cells were analyzed.

G Protein βγ Signaling Is Critical for Ethanol Effects.

Because amino acid sequences in the large intracellular loop of the GlyR modulate sensitivity to ethanol, it is possible that these sites are also involved in regulation of the GlyR by cell signaling. Recent studies have shown that ethanol activates specific transduction pathways, including Gβγ itself (15–17). Therefore, because it is known that the GlyR is modulated by Gβγ dimers (18, 24), we intended to establish a relationship between ethanol sensitivity and Gβγ modulation. Analyzing mutant GlyRs, we found a significant positive correlation between the sensitivity of the mutant receptors to 100 mM

ethanol and Gβγ activation, suggesting a role of Gβγ signaling for the ethanol effects (Table S2, Fig. 4, and Fig. S4). Thus, these results led to analyses toward the importance of Gβγ signaling for the ethanol effects on GlyRs. A common strategy for studying the involvement of Gβγ is to express proteins that bind with high affinity to these dimers. Thus, the overexpressed Gβγ-binding protein can buffer "free" Gβγ, thereby preventing its interaction with effectors. Therefore, we tested the ability of ct-GRK2 and ct-GRK3, known Gβγ scavengers (16, 24, 30), to attenuate the ethanol potentiation on wild-type GlyRs. Overexpression of these proteins significantly attenuated the potentiation induced by ethanol on wild-type GlyRs (Fig. 4 *A* and *B*), displaying significant decreases in the potentiation elicited by 1–100 mM ethanol, showing a smaller effect with 200 mM (Fig. 4*B*) and full potentiation with 500 mM ethanol (Fig. 4*C*). This indicates that the Gβγ signaling is only critical for ethanol effects within the pharmacological range, which agrees with the data in mutant receptors (Fig. 1). To study further the role of Gβγ signaling, we overexpressed several proteins having different affinities for the heterodimer. The overexpression of wild-type $G\alpha_t$ and $G\alpha_o$ subunits, having high affinity for Gβγ dimers (30, 31), also reduced ethanol effects on the GlyR (Fig. 4*D*). However, Gα Q-L mutants that displayed altered GTP hydrolysis and low Gβγ affinity (31) did not produce any significant attenuations (Fig. 4*D*). In addition, GlyRs tonically modulated by overexpression of $G\beta_1\gamma_2$ (18, 24) showed a lower ethanol potentiation. Finally, independent expression of $G\beta_1$ and $G\gamma_2$ did not affect the ethanol potentiation because both subunits are required for a functional Gβγ dimer (31) (Fig. 4*D*).

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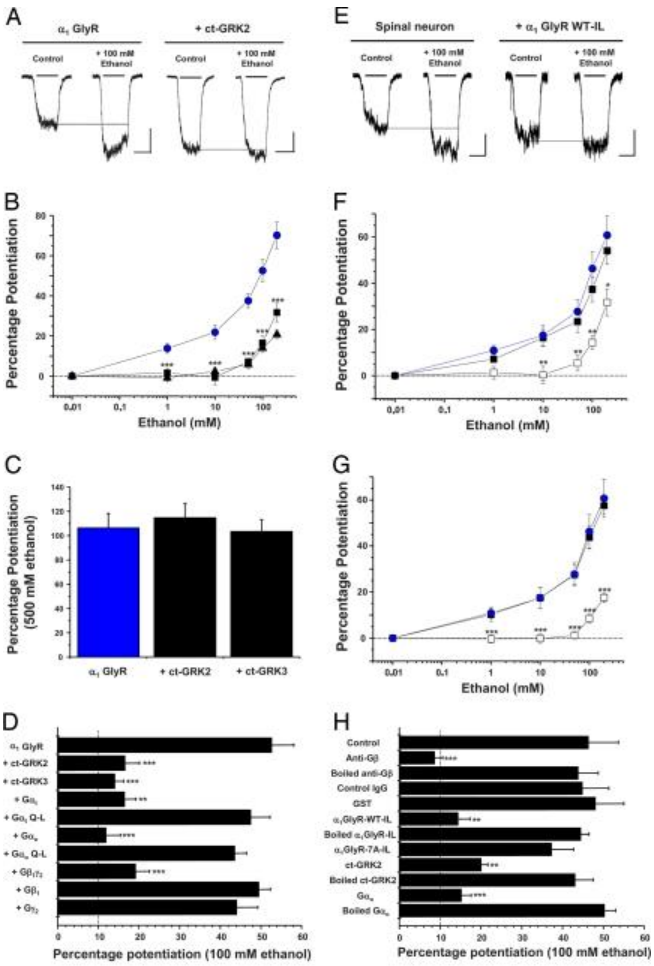

Fig. 4. Gβγ-mediated signaling is required for modulation of GlyRs by ethanol. (*A*) Current traces from HEK293 cells transfected with wild-type GlyRs, with or without ct-GRK2. (Scale bar: 200 pA, 1 s.) (*B*) Ethanol concentration–response curves of wild-type GlyRs in the absence (blue circles) or presence of ct-GRK2 (black squares) or ct-GRK3 (black triangles). Differences were significant (***, $P < 0.001$, ANOVA). (*C*) The graph summarizes the percentage potentiation elicited by 500 mM ethanol on wild-type GlyRs in the absence or presence of ct-GRK2-3. (*D*) Summary of the effects of 100 mM ethanol on wild-type GlyRs alone or in the presence of Gβγ scavengers (ct-GRK2-3), Gα subunits (wild-type or Q-L) or cotransfected with $Gβ_1$ and $Gγ_2$. Differences were significant (**, $P < 0.01$; ***, $P < 0.001$, ANOVA). (*E*) Current traces from spinal neurons in the presence or absence of intracellular $α_1$ GlyR TM3–4 loop. (Scale bar: 60 pA, 1 s.) (*F*) Ethanol concentration–response curves by using normal internal solution (blue circles) or dialyzed with the wild-type GlyR intracellular loop (IL-white squares) or mutant GlyR intracellular loop (7A-IL, black squares). Differences were significant (*, $P < 0.05$; **, $P < 0.01$; ANOVA) (*G*) Ethanol effects (1–200 mM) on neuronal GlyRs by using a normal internal solution (blue circles) or dialyzed with an anti-Gβ antibody (white squares) or heat-denatured anti-Gβ (black squares). Differences were significant (***, $P < 0.01$; ANOVA). (*H*) Summary of the effects of 100 mM ethanol on native GlyRs after intracellular dialysis with proteins that selectively bind Gβγ. The values represent the mean ±SEM of the percentage potentiation during coapplication of glycine and ethanol after 7–10 min of whole-cell recording. Differences were significant (**, $P < 0.01$; ***, $P < 0.001$, ANOVA).

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To confirm these results by using a neuronal background, we analyzed Gβγ signaling in mouse spinal neurons, which express endogenous $\alpha_1\beta$ GlyRs sensitive to ethanol (4, 5, 9). Interestingly, the ethanol potentiation was significantly attenuated when Gβγ signaling was antagonized by intracellular application of purified Gβγ scavenger proteins, such as ct-GRK2, $G\alpha_o$ subunits, or an antibody against Gβ subunits (Fig. 4 *G* and *H*). Additionally, ethanol potentiation was inhibited after dialysis of the GlyR intracellular loop (14 ± 3%, $n = 6$), known to bind Gβγ (18). Notably, a mutant version of the GlyR intracellular loop, in which the basic sequences ^{316}RFRRK and ^{385}KK implicated in the Gβγ binding were replaced by 7 alanines (18), did not significantly affect the ethanol potentiation (37 ± 5%, $n = 8$), confirming a specific role of these basic residues for the ethanol effects in a neuronal background (Fig. 4 *E*–*H*). Additional control experiments with GST or normal rabbit IgG, that did not interact with Gβγ, and heat-denatured Gβγ scavengers did not show any effect on the ethanol potentiation of the GlyR, supporting a specific high-Gβγ affinity requirement for the ethanol blockade effect. Altogether, these results support a key role for Gβγ in the potentiation of the glycine-activated current initiated by pharmacological ethanol concentrations.

Discussion

Our data permit the following conclusions with respect to the effects of ethanol on a member of the LGIC superfamily. First, mutations in intracellular residues significantly decreased the sensitivity only to pharmacologically relevant ethanol concentrations. Second, the high degree of specificity for ethanol supports the presence of a defined intracellular mechanism for ethanol potentiation in GlyRs, different from binding of alcohol within the GlyR structure. Third, because activation of signal transduction pathways can affect ethanol sensitivity, the strength of ethanol effects on GlyRs will depend on the state of intracellular G protein activation, which is also dependent on extracellular messengers. In this context, these conclusions appear to fit well with the ethanol actions reported on several LGIC receptors, which have been shown to be highly variable when concentrations <50 mM were used (2, 3, 32) and persistently modulated by high ethanol concentrations (1–3, 8, 32). Based on this concept, the intracellular G protein activation state could determine

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the sensitivity to pharmacological ethanol ranges, and other mechanisms, such as binding of ethanol to TM regions, may configure the sensitivity to higher alcohol concentrations.

At the molecular level, the wide range of ethanol effects on membrane receptors have been explained by means of 2 hypotheses: (*i*) direct binding of alcohol within the ion channel (8, 11) or (*ii*) indirect regulation of the channel caused by alcohol modulation of signal transduction elements (9, 13, 14). The former characterized mechanism found that specific transmembrane residues within the GlyR structure were necessary for ethanol effects (8, 26–29), concluding that alcohols and general anesthetics possess specific binding pockets inside the TM2 and TM3 domains of GlyRs (11). Nevertheless, the latter hypothesis postulates that ethanol modulates GlyR and $GABA_AR$ by indirect effects, mainly through the activation or inhibition of intracellular signals. For instance, it has been reported that G protein activation or inhibition modulates the ethanol sensitivity of neuronal GlyRs (9, 13, 14). In addition, other studies report that the $GABA_AR$ sensitivity can be regulated by PKC and PKA activation (3, 33, 34). The present work shows the existence of critical Gβγ-linked residues for ethanol regulation within the large intracellular loop of one member of the LGIC superfamily. Interestingly, mutations in these sites significantly decreased the sensitivity to pharmacologically relevant ethanol concentrations but did not alter the potentiation elicited by higher concentrations, which is likely related to binding of ethanol to other molecular sites (11). In addition, the sensitivity to other positive modulators, including general anesthetics and *n*-alcohols, was not affected, in contrast to previous studies with TM-mutated GlyRs (8, 26–29). In line with these data, free Gβγ buffering significantly attenuates the ethanol effects on wild-type recombinant and native GlyRs, linking the intracellular mutations to a known effector protein, widely expressed in mammalian cells. Thus, the present data allow us to propose a model in which pharmacological ethanol concentrations (≤100 mM) transiently activate Gβγ signaling, allowing the functional interaction between the dimer and the GlyR intracellular loop, thereby generating the enhancement of the glycine-activated current by ethanol (Fig. 5). Therefore, this working model provides a rational framework for the development of selective GlyR–Gβγ interaction inhibitors, which could be useful to study the ethanol effects on GlyRs in both in vitro and in vivo models. For example, this

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mechanism could be useful to understand the role of accumbal GlyRs in ethanol intake that is believed to be associated to alcoholism (20, 21) to elucidate the contribution of the ethanol-sensitive hypoglossal GlyRs to obstructive sleep apnea (10, 19).

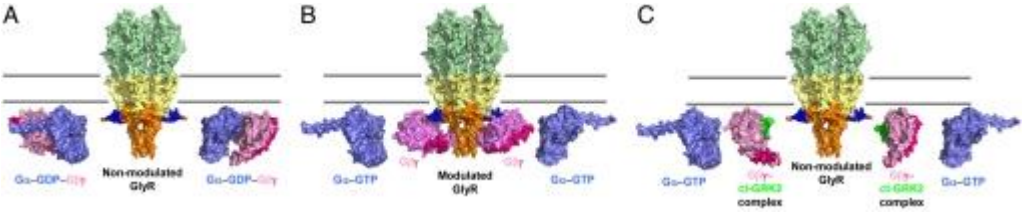

Fig. 5. Model for ethanol modulation of GlyRs. (*A*) In the nonmodulated state, G proteins are in their heterotrimeric conformation, and the GlyR is not functionally regulated by Gβγ. (*B*) Pharmacological ethanol concentrations increase free Gβγ dimer availability, which then interacts with the GlyR through basic residues within the intracellular loop. This converts the GlyR into a modulated state, producing the reversible enhancement of the glycine-activated current. (*C*) After overexpression of ct-GRK2, free Gβγ are bound by this high-affinity sequester and become unable to interact with the GlyR. Panels show pentameric GlyRs in a schematic plasma membrane (gray lines). The extracellular regions of the GlyR are shown in light green, TM domains in yellow, and the intracellular region in orange. The specific residues that interact with Gβγ are shown in dark blue (18). Gβ is drawn in magenta, Gγ in pink, Gα in blue, and ct-GRK2 in bright green.

In summary, these data support the hypothesis that ethanol potentiates a member of the LGIC superfamily via the Gβγ dimer and contributes to the understanding of the complex nature of alcohol effects on the human nervous system. These results, for example, might help to clarify the controversial effects of ethanol on $GABA_AR$ (3, 32), which is in line with the importance of intracellular signaling suggested by the key role of PKCε on the ethanol potentiation of $GABA_A$ receptors (34). In addition, this study raises the possibility for generating genetically modified animal models and selective Gβγ-based molecules (35) to block the ethanol effects on GlyR and other LGIC members, contributing to the development of drugs and therapies for ethanol-related medical problems.

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Methods

cDNA Constructs, Cell Culture, and Transfection.

The cDNA encoding the human GlyR α_1 subunit has been described in ref. 18. Mutations were inserted by using the QuikChange site-directed mutagenesis kit (Stratagene). GlyR amino acids were numbered according to their position in the mature protein. HEK293 cells and cultured spinal neurons were prepared as described (9, 18, 24).

Electrophysiology.

Whole-cell and single-channel recordings in outside-out configuration were performed as described (18, 24, 36). Further details are available in *SI Text*.

Molecular Modeling.

The GlyR model was constructed by using coordinates from *Torpedo* nAchR and acetylcholine-binding protein structures, as described in ref. 18. The structures of the heterotrimeric G protein and ct-GRK2 are based on coordinates from Lodowski *et al.* (37) and was constructed by using a protein docking protocol. Final images were generated with Pymol (38) and Gimp 2.3.

Data Analysis.

Statistical analyses were performed by using ANOVA and are expressed as mean ± SEM; values of $P < 0.05$ were considered statistically significant. Origin 6.0 (MicroCal) was used for all of the analyses and plots.

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Footnotes

The authors declare no conflict of interest.
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Supplementary Material

Supporting Information:

SI Text

Ethanol Effect on GlyR Is Positively Correlated with Gβγ Modulation.

Because amino acid sequences in the large intracellular loop of the GlyR modulate sensitivity to ethanol, it is possible that these sites are also involved in regulation of the GlyR by cell signaling. Recent studies have shown that ethanol activates specific transduction pathways, including G*βγ* signaling (1, 2). Therefore, because it is known that the human α_1 GlyR is modulated by G*βγ* dimers (3, 4), we intended to establish a relationship between ethanol sensitivity and G*βγ* modulation. To do so, we activated G proteins with a nonhydrolyzable analog of GTP, which increases the amplitude of the glycine-activated current by a G*βγ*-dependent mechanism (4). The analysis of the mutants revealed a highly significant positive correlation between the sensitivity of the mutant receptors to 100 mM ethanol and G*βγ* activation ($r^2 = 0.88$, $P < 0.0001$) (Fig. S4). However, no significant correlation was found between G*βγ* modulation and either a high (500 mM) concentration of ethanol or other GlyR modulators (Table S2). These analyses raise the possibility that pharmacological, but not higher, ethanol concentrations potentiate GlyRs through G*βγ* dimers.

Electrophysiology. Whole-cell recordings were performed as described, by using a holding potential of -60 mV (3, 4). For transfected HEK293 cells, the expression of GFP was used as a marker for positively transfected cells, and recordings were made after 18–36 h. The mouse spinal neurons recordings were performed after 10–13 days in vitro, a time in which the neurons express mainly $\alpha_1\beta$ GlyRs (5). Patch electrodes were filled with 140 mM CsCl, 10 mM BAPTA, 10 mM Hepes (pH 7.4), 4 mM $MgCl_2$, 2 mM ATP, and 0.5 mM GTP. The external solution contained 150 mM NaCl, 10 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM Hepes (pH 7.4), and 10 mM glucose. The amplitude of the glycine current was measured by using a short (1–2 s) pulse of glycine (EC_{10}) for each receptor studied. Strychnine (1μM)

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blocked all of the current elicited by wild-type and mutant glycine receptors (data not shown). Stock solutions of propofol (Tocris), alphaxalone (Steraloids), isoflurane (Baxter), trichloroethanol (Sigma), and *n*-alcohols (Merck and Sigma) were directly diluted in the extracellular solution. Modulators were coapplied with glycine, without preapplication, at room temperature (20–24°C). The ethanol effect on GlyRs was reported to depend on temperature, with a large break from linearity at temperatures <15°C (6). In this work, the ethanol potentiation was consistently detected at 20–24°C throughout the recordings, in agreement with our previous studies in cultured spinal neurons that showed strong ethanol effects on GlyRs recorded at room temperature or at 36°C. In all of the experiments, a brief pulse of 1 mM glycine was performed at the end of the recording period to test that the glycine concentration corresponded to the actual EC10 in each single experiment. Cells that displayed responses $<EC_5$ or $>EC_{15}$ were discarded. None of the G$\beta\gamma$ sequesters (ct-GRKs and Gα wild-type subunits) or Gα Q-L mutants produced significant alterations on the GlyR properties. For example, the EC_{50} in cells overexpressing ct-RK2 was 48 ± 2 μM, which was indistinguishable from control (46 ± 2 μM). Thus, the ethanol effect was tested in these cells by using the EC_{10} for the GlyR alone (15 μM). However, ethanol potentiation on G$\beta\gamma$-transfected cells were assayed by using an EC_{10} for that condition (10 μM, EC_{50} = 26 ± 2 μM) because we described that G$\beta\gamma$ shifted the concentration response curve to the left (3, 4). However, expression of Gβ or Gγ alone did not affect the GlyR properties, in agreement to previous studies (7–10). For spinal neuron recordings, ethanol sensitivity was assayed after 7–10 min of whole-cell recording. G$\beta\gamma$ scavengers were dissolved in the intracellular solution. None of the strategies used modified the glycine-evoked currents during the dialysis period; thus, for all of these experiments, a concentration equivalent to an EC_{10} for these spinal neurons (15 μM) was used (11, 12). Control IgG and anti-Gβ antibodies were from Santa Cruz Biotechnology; purified Gαo protein was from Calbiochem. The finding showing that the ethanol effect is observed under whole-cell and outside-out patch clamp configurations suggests that a great part of the critical components is tightly associated with the membrane. We did not perform gramicidin-perforated recordings to examine intracellular modulation because of their technical complexity (i.e., long recording times), the difficulties to test the dialysis of small-cell

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constituents (i.e., G proteins and G*βγ* sequesters) and because gramicidin could act as a binding protein to general anesthetics (13) or affect the kinetics of important membrane Na,K-ATPases (14) possibly by changes on phosphorylation.

The methodology for single-channel recordings in outside-out configuration has been published (4, 15). Briefly, patch pipettes were coated with R6101 elastomer (Dow-Corning) and had tip resistances of 7–15 MΩ after fire polishing. Cells were voltage-clamped at -50 mV, and the data were filtered (1-kHz low-pass 8-pole Butterworth) and acquired at 5–20 kHz by using pClamp software (Axon Instruments). Agonist and alcohol solutions were applied to cells by using a stepper motor-driven rapid solution exchanger (Fast-Step; Warner Instrument Corp.) Cells were maintained in extracellular medium containing 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM Hepes, 10 mM glucose (pH 7.4). The intracellular recording solution contained 140mM CsCl, 2 mM Mg-ATP, 10 mM BAPTA, and 10 mM Hepes (pH 7.2). In some membrane patches from mutant GlyRs, ethanol induced a small decrease in nPo that generated a slight negative effect. However, the average percentage change was not statistically different from control. The larger effect of ethanol on membrane patches compared with whole-cell recordings that we obtained in our experiments could be explained by a reduced availability for nonspecific binding of ethanol to hydrophobic pockets compared with an intact cell (16–17). However, it is important to note that larger G*βγ* effects on cell membrane patches, compared with whole-cell recordings, were reported by our group (4) and independently shown by Fischer and coworkers studying nAChRs (18). Thus, because the G*βγ* and ethanol are highly associated, the absence of a regulatory cytoplasmatic protein in the outside-out configuration also might produce this enhancement on the ethanol action.

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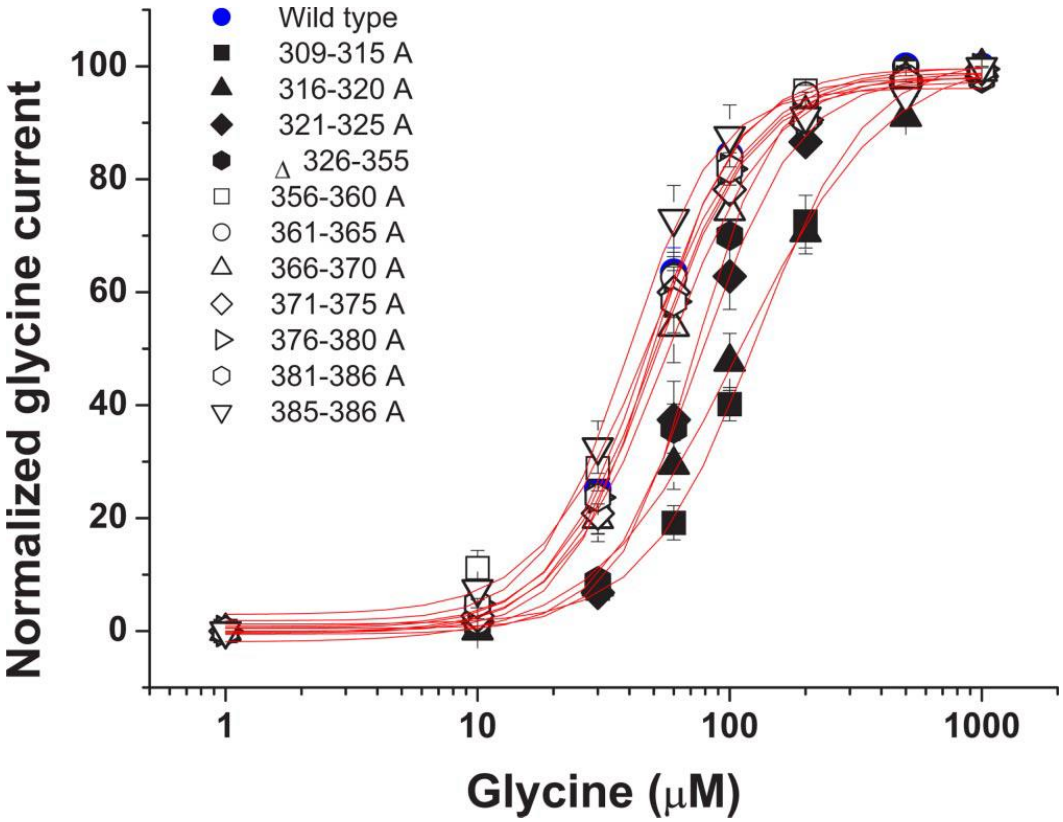

Fig. S1. Concentration–response curves of glycine-evoked currents in HEK293 cells transfected with human GlyRs with mutations or deletions in the TM3–TM4 intracellular loop. The curves were constructed from data after the application of 1, 10, 30, 60, 100, 200, 500, and 1,000μMglycine for 1–2 s to transfected HEK293 cells expressing wild-type or mutant GlyRs, recorded at a holding potential of -60 mV. The symbols represent the mean ±SEM obtained from normalized glycine-activated currents from at least 6 cells. Data were normalized to the peak amplitude obtained at a saturating concentration of glycine (1,000 μM). The 387–392A mutant GlyR was not included in the graph because no glycine-evoked current was detected under our experimental conditions.

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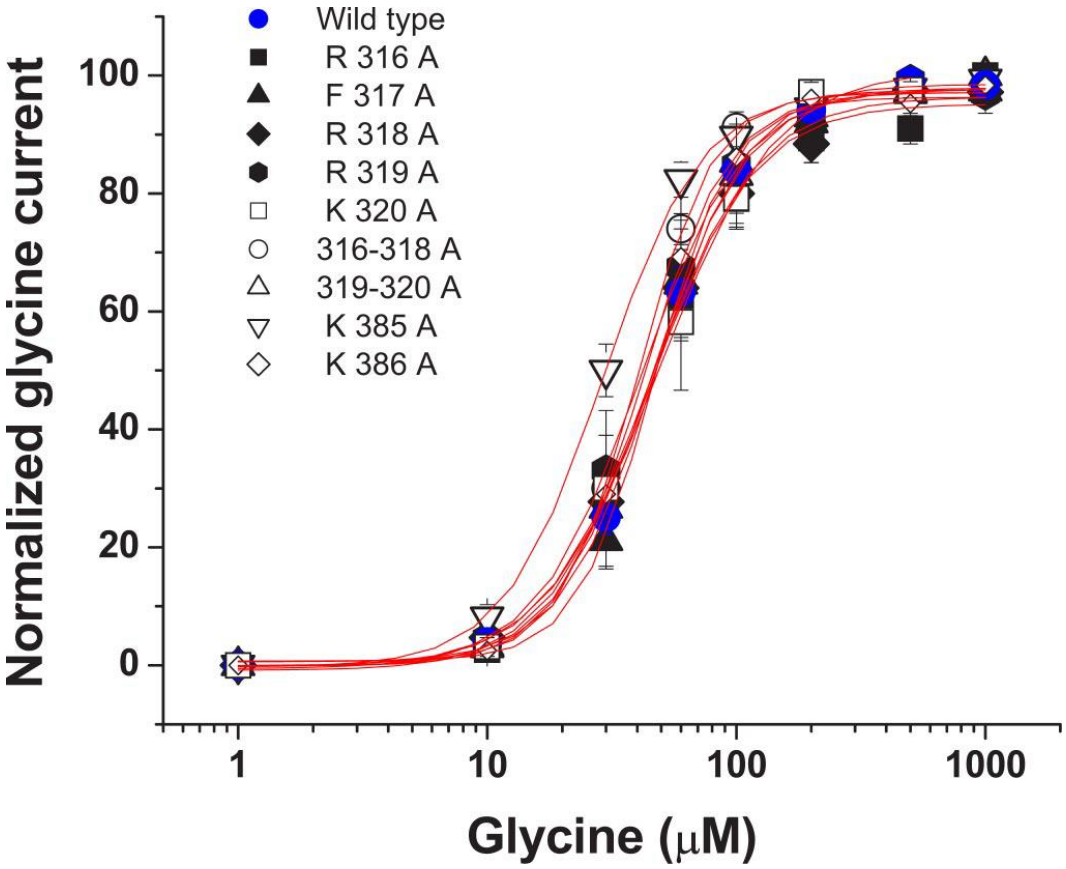

Fig. S2. Concentration–response curves of glycine-activated currents elicited by human GlyRs expressed in HEK cells with single or double alanine substitutions within the basic residue motifs important for the ethanol potentiation. The curves were constructed from data after the application of 1, 10, 30, 60, 100, 200, 500, and 1,000 μM glycine for 1–2 s to transfected HEK293 cells expressing wild-type or mutant GlyRs, recorded at a holding potential of -60 mV. The symbols represent the mean ±SEM obtained from normalized glycine-activated currents from at least 6 cells. Data were normalized to the peak amplitude obtained at a saturating concentration of glycine (1,000 μM).

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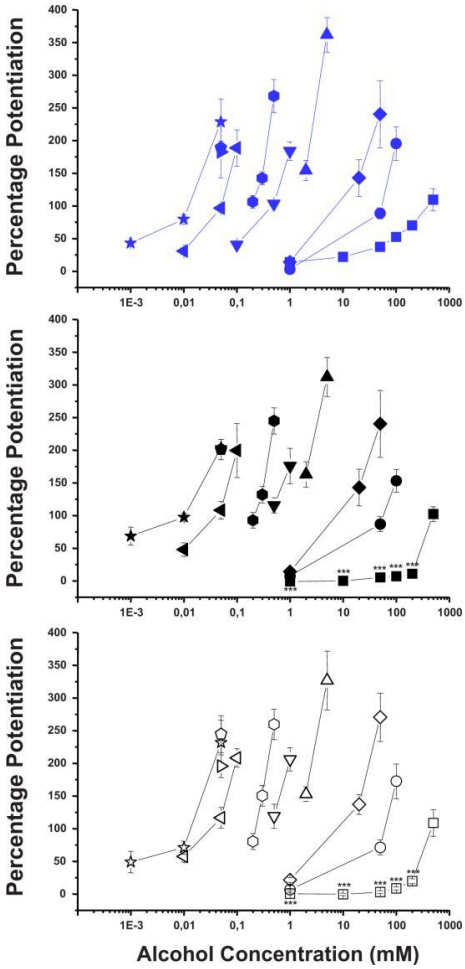

Fig. S3. Effects of *n*-alcohols on wild-type and mutated GlyRs. The graphs summarize the sensitivity to *n*-alcohols of wild-type and ethanol-resistant mutant GlyRs. Wild-type percentages are shown in blue, and 316–320A and 385–386A mutants are shown in black and white, respectively. For the analysis, ethanol (1–500 mM, squares), propanol (1–100 mM, circles), butanol (1–50 mM, diamonds), pentanol (2–5 mM, up-triangles), hexanol (0.5–1 mM, down-triangles), heptanol (0.2– 0.5 mM, hexagons), octanol (10–100 μM, left-sided triangles), nonalol (50 μM, pentagons), decanol (1–50 μM, stars), and undecanol (50 μM, right-sided triangles) were applied. The symbols represent the mean ±SEM obtained from at least 5 cells. Significant differences were found with ethanol concentrations <200 mM (***, $P < 0.001$, ANOVA).

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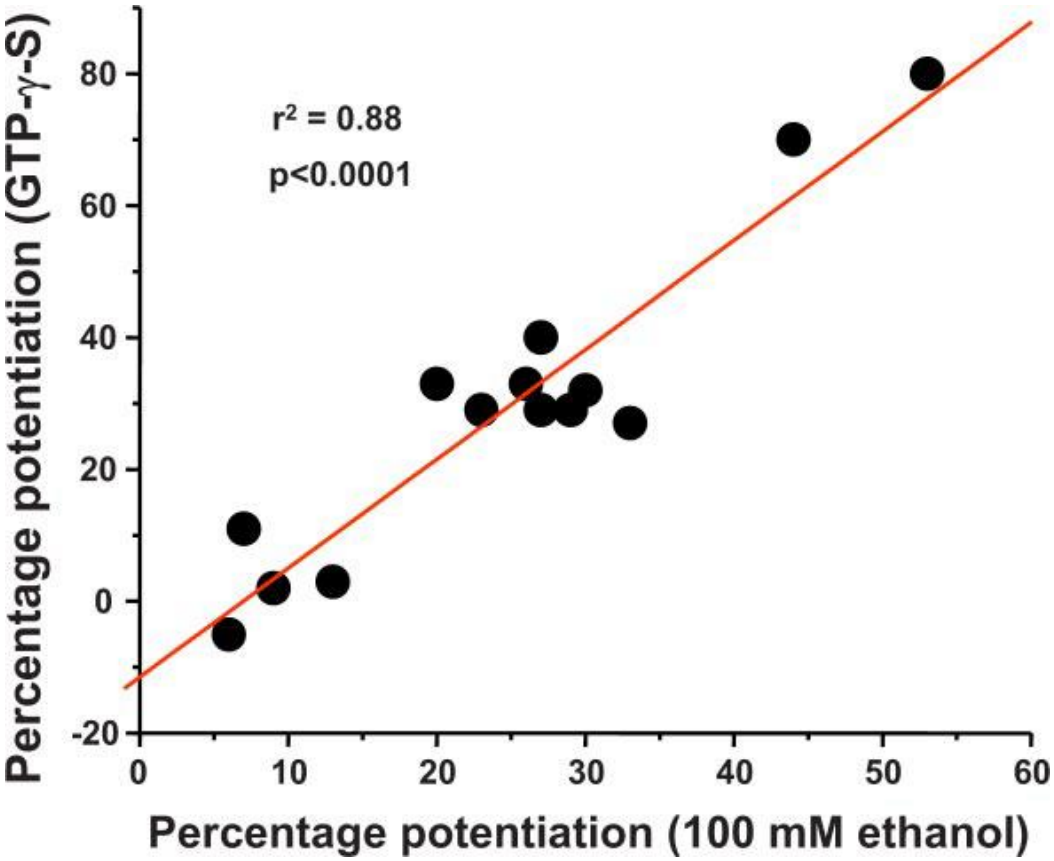

Fig. S4. Ethanol sensitivity correlates with G*βγ* modulation in wild-type and mutant GlyRs. Shown is a correlation plot between percentage potentiation in the presence of intracellular GTP*γ*S at minute 15 of whole-cell recording (3) and the potentiation induced by 100mMethanol. The r_2 value was highly significant, demonstrating a high correlation between G*βγ* modulation and ethanol sensitivity for each GlyR studied. The symbols represent the mean ±SEM obtained from at least 6 cells. For this analysis, the mutants from Fig. 1*C* were used.

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Table S1. Electrophysiological properties of human GlyRs mutated in the TM3–TM4 intracellular loop

Glycine receptor examined (sequences deleted or replaced with alanines)	EC_{50}, μM	n_H	I_{max}, pA	Calculated EC_{10}, μM	No. of cells
α_1 GlyR (wild type)	46 ± 2	2.5 ± 0.2	3,647 ± 324	15	23
309–315A (RQHKELL)	278 ± 20	1.5 ± 0.1	2,700 ± 450	108	6
316–320A (RFRRK)	110 ± 8*	1.5 ± 0.1*	1,787 ± 237*	30	15
321–325A (RRHHK)	77 ± 3*	2.3 ± 0.2	3,950 ± 758	17	5
Δ326–355 (EDEAGEGRFNSAYGMG-PACLQAKDGISVK)	76 ± 1*	2.5 ± 0.1	2,187 ± 371	17	6
356–360A (GANNS)	47 ± 3	1.9 ± 0.3	3,745 ± 567	17	6
361–365A (NTTNP)	49 ± 1	2.5 ± 0.1	4,745 ± 789	17	6
366–370A (PPAPS)	52 ± 2	2.1 ± 0.1	5,013 ± 513	18	6
371–375A (KSPEE)	53 ± 3	2.2 ± 0.2	4,560 ± 695	17	4
376–380A (MRKLF)	50 ± 2	2.1 ± 0.2	3,630 ± 755	18	5
381–386A (IQRAKK)	50 ± 3	2.2 ± 0.1	3,942 ± 693	17	6
387–392A (IDKISR)	No glycine-evoked current detected†				6
R316A	45 ± 3	2.1 ± 0.3	2,515 ± 278	15	8
F317A	47 ± 1	2.8 ± 0.2	3,641 ± 775	15	6
R318A	45 ± 2	2.1 ± 0.2	3,998 ± 911	15	7
R319A	41 ± 1	2.1 ± 0.1	3,064 ± 928	14	6
K320A	49 ± 3	1.9 ± 0.2	3,015 ± 482	15	5
316,318A	40 ± 1	2.2 ± 0.1	4,143 ± 653	14	8
319–320A	46 ± 2	2.2 ± 0.2	4,038 ± 810	15	11
385–386A	38 ± 2	2.5 ± 0.3	4162 ± 654	12	9
K385A	30 ± 2*	2.2 ± 0.2	4,423 ± 710	11	6
K386A	42 ± 1	2.5 ± 0.1	4,580 ± 747	15	8

Values are given as mean ± SEM. Values were fitted to the equation $I_{glycine} = I_{max}[glycine]^{n_H}/([glycine]^{n_H} + [EC_{50}]^{n_H})$ using a computer software.
*Significant difference of $P < 0.05$, ANOVA. The calculated EC_{10} concentration for each GlyR was used to test the ethanol sensitivity and the effects of all other modulators studied.
†These parameters could not be quantified because currents were either nonexistent or too small to measure under our experimental conditions.

Table S2. Ethanol sensitivity correlates with Gβγ modulation in wild-type and mutant GlyRs expressed in HEK293 cells

Allosteric modulators	r^2	P
Alcohols		
Ethanol (100 mM)	0.88	<0.0001*
Ethanol (500 mM)	0.07	0.62
Propanol (50 mM)	0.05	0.68
Butanol (20 mM)	0.23	0.33
General anesthetics		
Isoflurane (500 μM)	0.25	0.31
Propofol (50 μM)	0.06	0.64
Other modulators		
Zn^{2+} (1 μM)	0.24	0.33
Trichloroethanol (3 mM)	0.16	0.43
Alphaxolone (50 μM)	0.08	0.57

The table summarizes r^2 values obtained from correlation plots between the percentage potentiation with intracellular GTPγS and the effect of different allosteric modulators for GlyRs. The potentiation induced by 100 mM ethanol showed a highly significant r^2 value, demonstrating a positive correlation between Gβγ modulation and ethanol sensitivity for each GlyR studied. No significant correlation was found between Gβγ modulation and potentiation elicited by all other modulators tested, including general anesthetics and *n*-alcohols. Noteworthy, when 500 mM ethanol was considered, no correlation was found, indicating that the Gβγ modulation is only relevant for the ethanol effects at behaviorally modifying concentrations.